

AN IMMUNOLABELLING TECHNIQUE TO TRACK SPERM FROM DIFFERENT MATES IN THE FEMALE REPRODUCTIVE ORGANS OF TERRESTRIAL GASTROPODS

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ABSTRACT

The mechanisms of sperm transfer, storage, utilization and digestion are crucial for understanding processes of postcopulatory sexual selection. Previous studies analysing postcopulatory processes have generally focused only on the ultimate outcome of the interactions between male and female sexual selection (paternity patterns). For a mechanistic understanding of the fate of received sperm and the involved patterns of postcopulatory sexual selection new techniques are required. Here we present an improved immunolabelling technique to track the fate of 5-bromo-2'-deoxyuridine (BrdU)-labelled sperm in the female reproductive organs of gastropods. The technique was tested in individuals of the simultaneously hermaphroditic land snail *Arianta arbustorum* (Linnaeus, 1758). We determined the percentage of labelled sperm in spermatophores delivered and assessed the reliability of detecting labelled sperm in the spermatheca (sperm storage organ) and bursa copulatrix (sperm digestion organ) of the recipients. In our tests, the proportion of sperm labelled among the sperm produced by an individual averaged 99.3%. Furthermore, labelled sperm could be consistently visualized in both the sperm storage and sperm digestion organ of all recipients examined. Combined with a traditional sperm staining technique, we showed that the BrdU-labelling technique allows us to distinguish between sperm from two males in the female reproductive tract of double-mated snails. In a controlled growth experiment, we found that repeated BrdU-application slightly retarded shell growth of juvenile snails, but did not influence their adult size, survival, number of sperm delivered and mating frequency. The technique presented could be adjusted to other gastropod species providing insight into mechanisms of sperm competition and cryptic female choice.

Key words: *Arianta arbustorum*, female reproductive organs, Gastropoda, immunolabelling, postcopulatory sexual selection, spermatheca, sperm digesting organ, sperm storage.

INTRODUCTION

Sexual selection continues after copulation in many animal species by way of sperm competition and female sperm manipulation (Birkhead & Møller, 1998). Sperm competition occurs when spermatozoa from different males compete in the reproductive tract of a female for the fertilization of her eggs (Parker, 1970). In a variety of species, females have a physiologically and morphologically complex reproductive system, which may enable them to influence or even control offspring paternity by postcopulatory sperm storage and selective sperm use (frequently summarized as cryptic female choice; Eberhard, 1996). Disentan-

gling patterns of sperm storage, sperm use and sperm digestion is the key to understand mechanisms of sperm competition and cryptic female choice (Birkhead & Møller, 1998). So far, most studies infer these patterns from paternity data, which only reveal the ultimate outcomes of the interactions between male and female reproductive processes (Schärer et al., 2007). With a mechanistic understanding of the fate of received sperm, and the involved patterns of postcopulatory sexual selection, we can better understand the evolution of male and female reproductive morphology and physiology.

Different approaches have been developed to track the donor's sperm in the recipient. Using radiolabelled nucleotides, which result in

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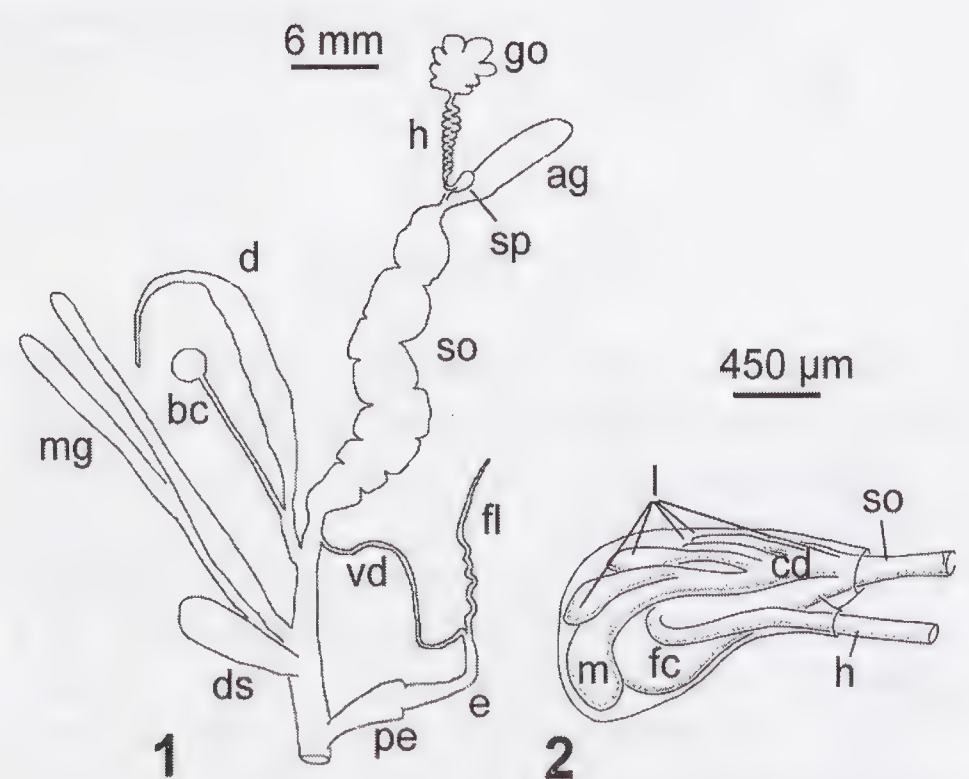
a specific labelling of the DNA of the donor's sperm, the position of sperm in the female tract can be located by scintillation counting or autoradiography (e.g., Beeman, 1970; Nollen, 1975; Bishop, 1996). However, this approach has several limitations due to radioactivity and laboratory facility requirements. Recently, insertions of genes for a green-fluorescent protein in the genome of *Drosophila melanogaster* have been used to distinguish sperm from different males (Civetta, 1999; Manier et al., 2010). The successful application of this method requires an extensive knowledge of the genome of the target species that is currently only available for a few species. Microsatellite competitive PCR is another technique for quantifying the relative number of sperm from different males stored in the spermatheca of multiple mated females (Bussi re et al., 2010; Hall et al., 2010). However, this technique is of limited use when postcopulatory female choice (sperm storage or digestion) is based on the genetic heterozygosity or complementarity of the mating males (e.g., Minoretta et al., 2011).

In recent years, immunocytochemical techniques have been developed to visualize particular types of cells. For example, 5-bromo-2'-deoxyuridine (hereafter called BrdU), a synthetic analogue of the nucleic acid component thymidine, is incorporated into newly synthesized nucleic acids by substituting for thymidine. BrdU can be visualized with immunocytochemical staining methods. This technique is commonly used for cancer cell detection in humans and in cell development research (Soames et al., 1994). In a few gastropod species, BrdU has been used to investigate brain cell development and the immune defense system (Zakharov et al., 1998; Gorbushin & Iakovleva, 2006). Sch rer et al. (2007) and Janicke & Sch rer (2009) used the BrdU-immunocytochemical approach to track sperm in the transparent flatworm *Macrostomum lignano*. Individuals of this free-living flatworm were labelled by incubation in BrdU-enriched artificial seawater. There was a considerable loss of flatworms in the staining process (36%; Sch rer et al. 2007), and no data on the percentage of labelled sperm have been reported. In the present study, we further developed the BrdU-labelling method to track sperm received in the female organs of single and double-mated individuals of the simultaneously hermaphroditic terrestrial pulmonate *Arianta arbustorum* (Linnaeus, 1758).

In stylommatophoran gastropods, the site of storage (= spermatheca) of received sperm

(allosperm) shows an enormous interspecific variability in morphology and number of tubules (reviewed in Baur, 2010). For example, *Oxychilus draparnaudi* (Beck 1837) and *Bradybaena fruticum* (M ller 1774) have a single spermathecal tubule. In *Succinea putris* (Linnaeus, 1758) two spermathecal tubules occur, and 34 tubules have been recorded in the spermatheca of *Drymaeus papyraceus* Mawe, 1823. There is also a considerable within-species variation in the number of spermathecal tubules (e.g., 3–5 in *Helix pomatia* Linnaeus, 1758; 4–19 in *Cornu aspersum* M ller, 1774; Evanno & Madec, 2007; Koentzopoulos & Staikou, 2007). As a consequence of the large intraspecific variation in the number of spermathecal tubules, different individuals have different possibilities to store allosperm from more than one mating partner. Mixing of sperm from different mates is more likely in a less structured spermatheca, whereas a large number of tubules potentially allows better separation of spermatozoa from different mates.

In stylommatophoran gastropods, the received sperm travel through the spermoviduct to the spermatheca (Figs. 1, 2), where a minor fraction is stored (Lind, 1973; Rogers & Chase, 2001). The majority of sperm, however, are directed to the sperm-digesting bursa copulatrix (Beese et al., 2006a; Fig. 1). In the spermath-



FIGS. 1, 2. Schematic representations of reproductive system of *A. arbustorum*. FIG. 1: Line drawing of reproductive system; FIG. 2: Line drawing of spermatheca; Abbreviations: ag albumen gland, bc bursa copulatrix, cd common duct, d diverticulum, ds dart sac, e epiphallus, fc fertilization chamber, fl flagellum, go gonad, h hermaphroditic duct, l lateral tubules, m main tubule, mg mucous glands, pe penis, sp spermatheca, so sperm oviduct, vd vas deferens.

eca, allosperm are stored for long periods (up to four years; Baur, 1998). Sperm survive best when attached to the walls of spermathecal tubuli (Chase & Darbyson, 2008).

The aim of our study was to further develop the BrdU-technique to distinguish between spermatozoa from two donors (one labelled, the other unlabelled) stored in the spermatheca of *A. arbustorum*. We injected BrdU into growing individuals of *A. arbustorum* and conducted several tests to assess the reliability of sperm labelling in the donor snail, to distinguish between labelled and unlabelled sperm and to demonstrate labelled sperm in different organs of the sperm-receiving mating partner. We also examined whether repeated BrdU-injections influence shell growth and adult size of snails. Finally, we present a first image showing the differential storage of sperm from two donors in the highly structured spermatheca of a double-mated individual of *A. arbustorum* and describe a method to quantify the relative amounts of sperm stored from both donors.

MATERIALS AND METHODS

Study Organism

Arianta arbustorum is common in moist habitats of northwestern and central Europe (Kerney & Cameron, 1979). The snail has determinate growth (shell width of adults 17–22 mm; Baur, 1984). Individuals become sexually mature at 2–4 years and adults live another 3–4 years (Baur & Raboud, 1988). Mating is random with respect to shell size, different degrees of relatedness and mating history (Baur, 1992; Baur & Baur, 1997; Kupfernagel & Baur, 2011). Copulation is reciprocal; both snails transfer simultaneously one spermatophore (Baur, 2007). The number of sperm delivered varies considerably (803,000–3,969,000), with an average of 2,185,000 (Baur et al., 1998). However, studies in other helicid snails revealed that only a minor fraction of sperm received are stored in the spermatheca (0.02–0.10%; Lind, 1973; Rogers & Chase, 2002). Snails need at least eight days to replenish their sperm reserves after a successful copulation (Locher & Baur, 1999). Sperm length varies from 878 to 939 μm in *A. arbustorum* (Fig. 3; Minoretti & Baur, 2006; Minoretti et al., 2013).

The sperm storage organ of *A. arbustorum* shows a considerable variation in the number of blind-ending spermathecal tubules (2–9). The tubules unite into a common duct that

opens into the fertilization chamber (Fig. 2; Baminger et al., 2000; Beese et al., 2009). Incoming sperm are not equally distributed among all spermathecal tubules, suggesting that sperm from different partners might be stored separately (Baur, 1994; Haase & Baur, 1995; Bojat & Haase, 2002). The musculature surrounding the spermathecal tubules is arranged in a complex three-dimensional network (Bojat et al., 2001a). If there were a selective activation of the muscles of each tubule (which has not yet been examined), this might allow the animal to selectively utilize sperm stored in single tubules and thus promote a selective fertilization of eggs (Baur, 2010).

In *A. arbustorum*, the dominant mode of reproduction is cross-fertilization. The snails mate repeatedly in the course of a reproductive season, and fertile sperm from different partners can be stored for more than one year in the sperm storage organ (Baur, 1988). There is a probability of 5–8% that a copulation will not lead to fertilization of eggs (no sperm or spermatophore transfer or transfer of infertile sperm; Chen & Baur, 1993). In the absence of potential mating partners, a low rate of self-fertilization can be observed both in breeding experiments and in natural populations (Chen, 1994; Kupfernagel et al., 2010).

Snail Sampling and Maintenance

Juvenile individuals of *A. arbustorum* (shell width: 8–12 mm) were collected in the sub-alpine forest near Gurnigelbad, Switzerland (46°45.5'N, 7°27.5'E, elevation 1,230 m a.s.l.) on 14 May 2009 and 30 April 2012, 2–4 weeks after hibernation. Snails were kept individually in transparent beakers (8 cm deep, 6.5 cm in diameter) lined with moist soil (approximately 4 cm) at 19°C with a light:dark cycle of 16:8 h.

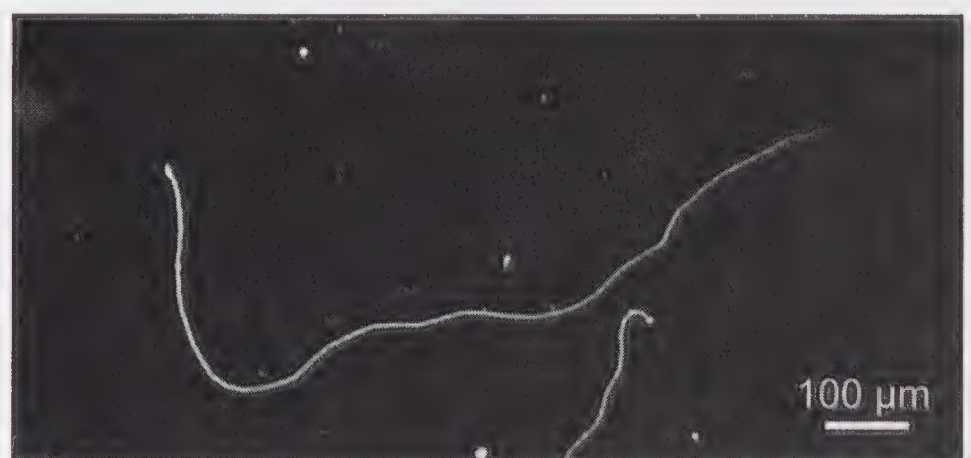


FIG. 3. Spermatozoon of *A. arbustorum* (unlabelled; microscopical phase contrast image). In this species, sperm measures 878–939 μm in length of which the head accounts for 15–25 μm (see Minoretti & Baur 2006).

Fresh lettuce was provided twice a week and at the same time the beakers were cleaned. In the wild, juvenile snails of the size range considered in this study may attain adulthood within 4–6 months and reproduce in the following year(s). Under the more favourable laboratory conditions, the juvenile snails reached sexual maturity, indicated by the formation of a reflected lip at the shell aperture, after 2–3 months. The snails were marked individually by writing numbers on their shells with a waterproof felt-tipped pen on a spot of correction fluid (Tipp-Ex). The animals showed no visible reaction to the marking procedure.

Sperm Labelling

Juvenile snails were randomly assigned either to a treatment group (hereafter called BrdU-labelled) or to a control group (hereafter called unlabelled). Individuals of the BrdU-labelled group received a BrdU injection (5-bromo-2'-deoxyuridine, Roth AG, Arlesheim, Switzerland; 1% in Ringer solution) every second week until they reached sexual maturity and this was continued afterwards, now as adults. Using a hypodermic syringe (needle size: 100 µm; U-100, BD Medical, Le Pont-de-Claix, France), 100 µl of solution was injected into the foot muscle, next to the retractor muscle and shell chamber. Absorption of BrdU due to skin immersion and/or injection has already been described in other invertebrate species (Zakharov et al., 1998; Gorbushin & Iakovleva, 2006; Schärer et al., 2007). BrdU is mainly incorporated into newly synthesized nucleic acid. Consequently, to ensure that all of the continuously produced sperm are labelled, BrdU-injections were repeated at intervals of two weeks over the course of the experiments.

BrdU Immunolabelling and DAPI Staining

To examine the fate of sperm in the female reproductive organs of the recipient, unlabelled snails were allowed to mate with BrdU-labelled individuals (for details see below). After successful copulation, recipients were killed by deep freezing (−80°C) and their female reproductive organs were dissected out and fixed for 2–4 h in paraformaldehyde (4%). Next, the reproductive organs were rinsed in distilled water (30–60 min), incubated in 50% ethanol (15 min) and finally stored in 70% ethanol. For immunolabelling and structural analyses, the

organs were embedded in paraplast, serially sectioned at 8 µm and positioned on Histo-Bond® microscope slides. One microscope slide carried 3–5 sectioned tissue slices. The slides were then deparaffinized in xylene and rehydrated through graded ethanol solutions to phosphate-buffered saline (PBS).

The tissue slides were treated with 0.5% trypsin (in EDTA; Invitrogen AG, Basel, Switzerland) for 20 min at 37°C, washed 5 min in PBS-T (PBS with 1% Triton X-100; Roth AG, Arlesheim, Switzerland), followed by a treatment with 2 N HCl for 30 min at room temperature to denature the DNA. After rinsing in PBS-T, enzyme activity and DNA denaturation were blocked with BSA-T (= PBS-T with 1% albumin fraction V; Roth AG, Arlesheim, Switzerland) for 15–30 min at 37°C. Tissue was incubated overnight at room temperature (alternatively for 4–5 h at 37°C) in a mouse monoclonal antibody directed against single-stranded DNA containing bromo-associated-uridine (BU-33, diluted 1:50 in BSA-T; Sigma, Missouri, USA). After this, the slides were washed four times in PBS-T, followed by 15 min in goat serum (G9023; Sigma, Missouri, USA) at 37°C to block unspecific binding, followed by a 1 h incubation at 37°C with a goat-anti-mouse FITC-conjugated secondary antibody (1:100, F5897; Sigma, Missouri, USA) directed specifically against the BU-33 antibodies. With the incubation of the second antibody the cell nuclei marker DAPI (1:500; Roth AG, Arlesheim, Switzerland) was added for counterstaining. DAPI labels the DNA of all cells and can therefore be used to compare the number of BrdU-labelled sperm with the total number of sperm occurring in a reproductive organ. Following our protocol, DAPI-incorporation was inhibited in BrdU-labelled sperm. This fact can be used to recognize unlabelled sperm in recipient snails.

After final washing steps (three times with PBS-T), tissue slides were embedded in PBS-glycerol (ratio 1:1) with the anti-microbial substance Thimerosal (0.005%; Roth AG, Arlesheim, Switzerland) and coverslipped. After the staining procedure, the slides were stored at 4°C for up to seven days in darkness to prevent fading of fluorescence. For fluorescence visualization of BrdU-labelled sperm and cells, a microscope (Olympus AX-70) with magnifications ranging from 40 to 400 was used. For digital photography the imaging software SPOT version 4.6 (Diagnostic Instruments Inc., Michigan, 2006) was applied.

Proportion of Labelled Sperm

Two sets of mating trials were conducted to assess the percentage of labelled sperm among all sperm produced by individuals treated with BrdU. First, we assessed the proportion of labelled sperm delivered during the first copulation. BrdU-labelled individuals were allowed to mate with virgin unlabelled individuals (functioning as recipients) for the first time. In three recipients, the spermatophore received was dissected out immediately after copulation (described below). Second, we examined the percentage of labelled sperm produced by donor snails after the first copulation. Mated BrdU-labelled snails were allowed to replenish their autosperm reserves for 7–10 days. The snails received a further BrdU-injection one to two days after their first copulation. These snails were again paired with virgin unlabelled individuals serving as recipients and the spermatophores delivered by the BrdU-labelled snails were dissected out of the recipients. To examine whether labelled and control snails differ in number of sperm delivered during copulation, we also paired unlabelled individuals with unlabelled recipients ($n = 6$) and dissected out the spermatophores.

Sperm were extracted from the obtained spermatophores as described in Locher & Baur (1997). The sperm mass delivered by each BrdU-labelled individual was divided into two equal aliquots. The first was used for immunostaining. Sperm were stored at -20°C until preparation for immunostaining. From the sperm solution, 2 μl were loaded on HistoBond® microscope slides (Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany) and air-dried for immunostaining. After immunostaining, we counted the number of labelled (BrdU with conjugated fluorescence marker FITC) and unlabelled (DNA with fluorescence marker DAPI) sperm on images of four randomly chosen slide sections of each spermatophore. For sperm counting on the digital photographs, the open source programme ImageJ version 1.44 (<http://rsbweb.nih.gov/ij/>) was used. This software facilitates manual counting and particle analysis from images. Based on the counts of labelled and unlabelled sperm, the percentage of labelled sperm delivered in each spermatophore was calculated. The repeatability of this technique was assessed by using multiple measurements ($n = 4$) in randomly-chosen sections ($n = 3$) on photographs following Lessells & Boag (1987). The second aliquot was used for estimating the total number of sperm delivered with the spermatophore, following Locher & Baur (1997).

To examine whether sperm from two donors (one BrdU-labelled and the other unlabelled) can be distinguished, we compared the immunostaining of BrdU-labelled sperm and unlabelled sperm in a 1:1 mixture of sperm (each 1 μl) from both donors on microscope slides.

BrdU-Labelled and Unlabelled Sperm in the Female Organs of the Recipient

Mating trials were conducted to examine the reliability of immunostaining of labelled sperm in the female organs of unlabelled sperm receivers. The spermatheca and bursa copulatrix of six unlabelled individuals that mated with a BrdU-labelled snail were dissected out at 48 h after copulation. This period of time allows sperm to leave the spermatophore and to reach the sperm storage organ. The remaining sperm are transported into the bursa copulatrix. None of the snails had laid eggs within 48 h after copulation. In three pairs, the BrdU-labelled partner mated for the first time, in the other three pairs for the second time.

Spermatheca and bursa copulatrix were embedded in paraplast, serially cross-sectioned at 8 μm and labelled with FITC and DAPI. By combining information from 2–3 successive tissue slices, it is possible to estimate the number of sperm stored (sperm heads measure 15–25 μm ; Minoretti & Baur, 2006). However, it is extremely labour-consuming to count all the sperm stored in the organ. We therefore decided to quantify results as the percentage of labelled sperm in relation to all sperm in the organ (see below). To check for the occurrence of auto-fluorescence, the sperm, spermatheca and bursa copulatrix of two unlabelled snails that copulated with an unlabelled partner were investigated as described above.

Finally, to visualize the pattern of sperm storage in the spermatheca of double-mated *A. arbustorum*, virgin unlabelled individuals were allowed to copulate with two partners, first with an unlabelled snail and then with a BrdU-treated snail. The recipients were killed by freezing (-80°C) 48 h after copulation. The spermatheca were cross-sectioned at 8 μm and labelled with FITC and DAPI as described above.

Quantification of BrdU-Labelled and Unlabelled Sperm in the Spermatheca

To obtain a quantitative estimate of the relative amount of sperm stored from each of the two donors in the spermatheca of a double-mated individual of *A. arbustorum*, the areas covered

TABLE 1. Percentage of BrdU-labelled sperm and total number of sperm in spermatophores delivered by BrdU-treated individuals of *A. arbustorum* during the first and second copulation.

Snail ID	First/second copulation*	Labelled sperm (%) Mean (range)†	Total number of sperm delivered
L1	first	98.3 (96.7–100.0)	1.25 x 10 ⁶
L16.1	first	99.1 (97.9–100.0)	1.00 x 10 ⁶
L11	second	99.4 (98.5–100.0)	1.69 x 10 ⁶
L16.2	second	99.9 (99.4–100.0)	0.96 x 10 ⁶
L19	second	99.7 (99.1–100.0)	1.51 x 10 ⁶

* First: sperm were produced during growth before the first copulation; second: sperm were also produced between the two copulations.
† Labelled sperm were counted in each of three randomly chosen sections (each containing 29–488 immunostained sperm) from four different images per spermatophore.

by either type of sperm (BrdU-labelled and unlabelled) in the spermathecal tubules were assessed. Every tissue slice of the cross-sectioned spermatheca of the recipient was photographed and inspected for sperm. Only photographs with visible sperm heads were selected (= informative slices). The areas covered by sperm heads of either type on a slice were determined by counting the number of pixels for each sperm head type using Adobe Photoshop CS3 (Adobe Systems Inc., USA, 2007). To avoid counting the same sperm head twice in successive photographs, every fourth slice was examined. This necessary restriction resulted in 10 and 23 informative slices obtained from the spermatheca of two double-mated individuals. The number of informative slices varies among individuals because of size differences in the sperm storage organ and because the orientation of the spermatheca cannot be controlled before it is embedded in paraplast. For the estimate of the percentages of sperm stored from both donors, the sum of pixels of 10 slices per spermathecae was considered. Reducing the number of informative slices from 23 to 10 did not substantially change the percentages of sperm stored from either donor.

Effect of BrdU on Snail Growth and Adult Size

To examine whether repeated BrdU-injections influence shell growth, adult size and survival of *A. arbustorum*, the shell width of BrdU-labelled (n = 18) and control snails (n = 17) were measured three times to the nearest 0.1 mm using vernier calipers (immediately after sampling, after 42 and 134 days in summer 2012). Shell

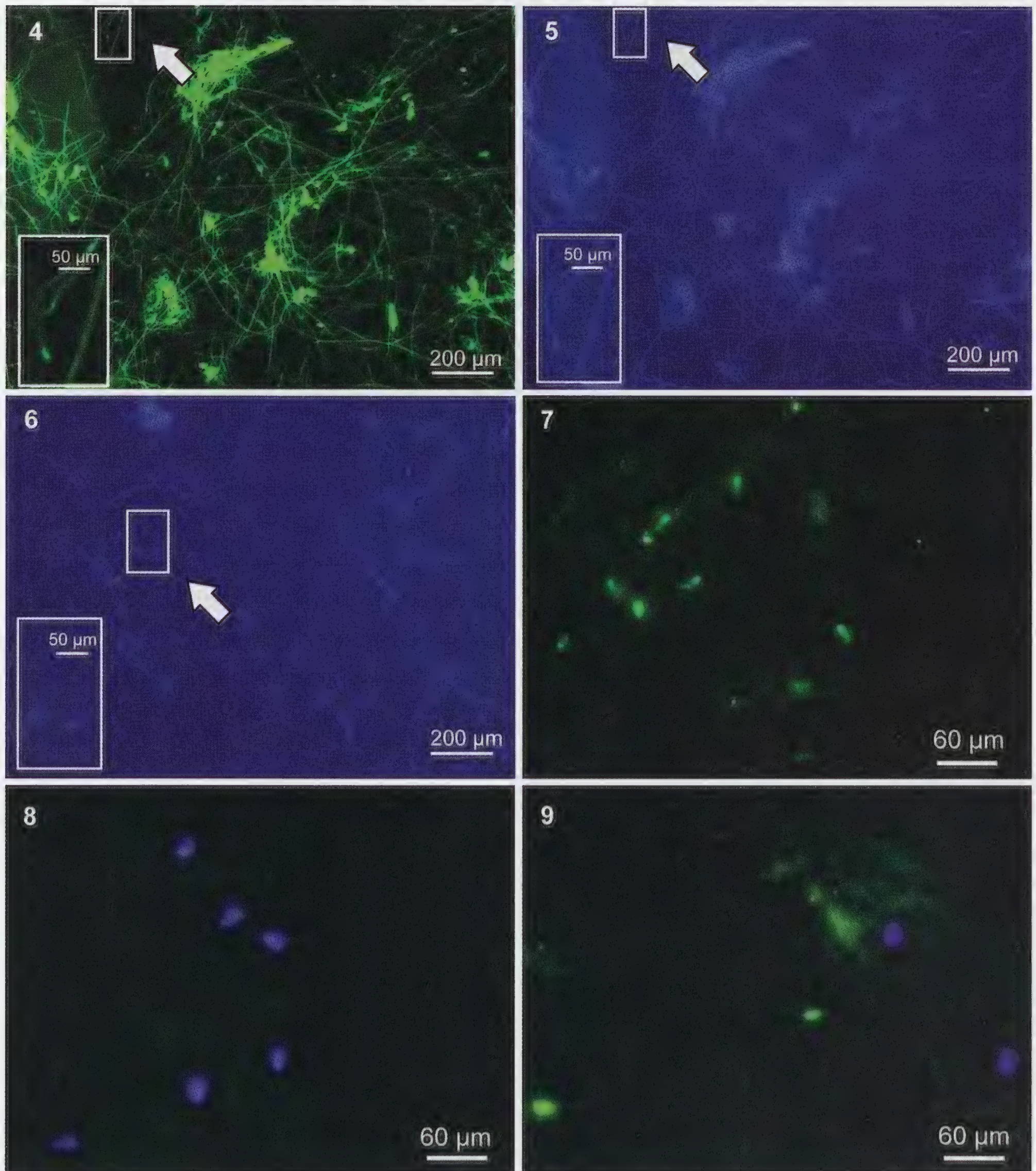
growth was expressed as growth rate (shell width increase per day in mm). Snail maintenance was as described above. Labelled snails received 10 BrdU-injections (each 100 µl of 1% solution) within 146 days (the time elapsed between two injections averaged 16.2 days). At the end of the experiment (after 160 days), the adult shell width of all individuals that had completed shell growth was measured and the number of surviving snails was determined for both groups. We also recorded the mating success of snails that reached sexual maturity.

All statistical analyses were conducted using PASW® statistics 19.0 core system (SPSS Inc., 2010).

RESULTS

Proportion of BrdU-Labelled Sperm

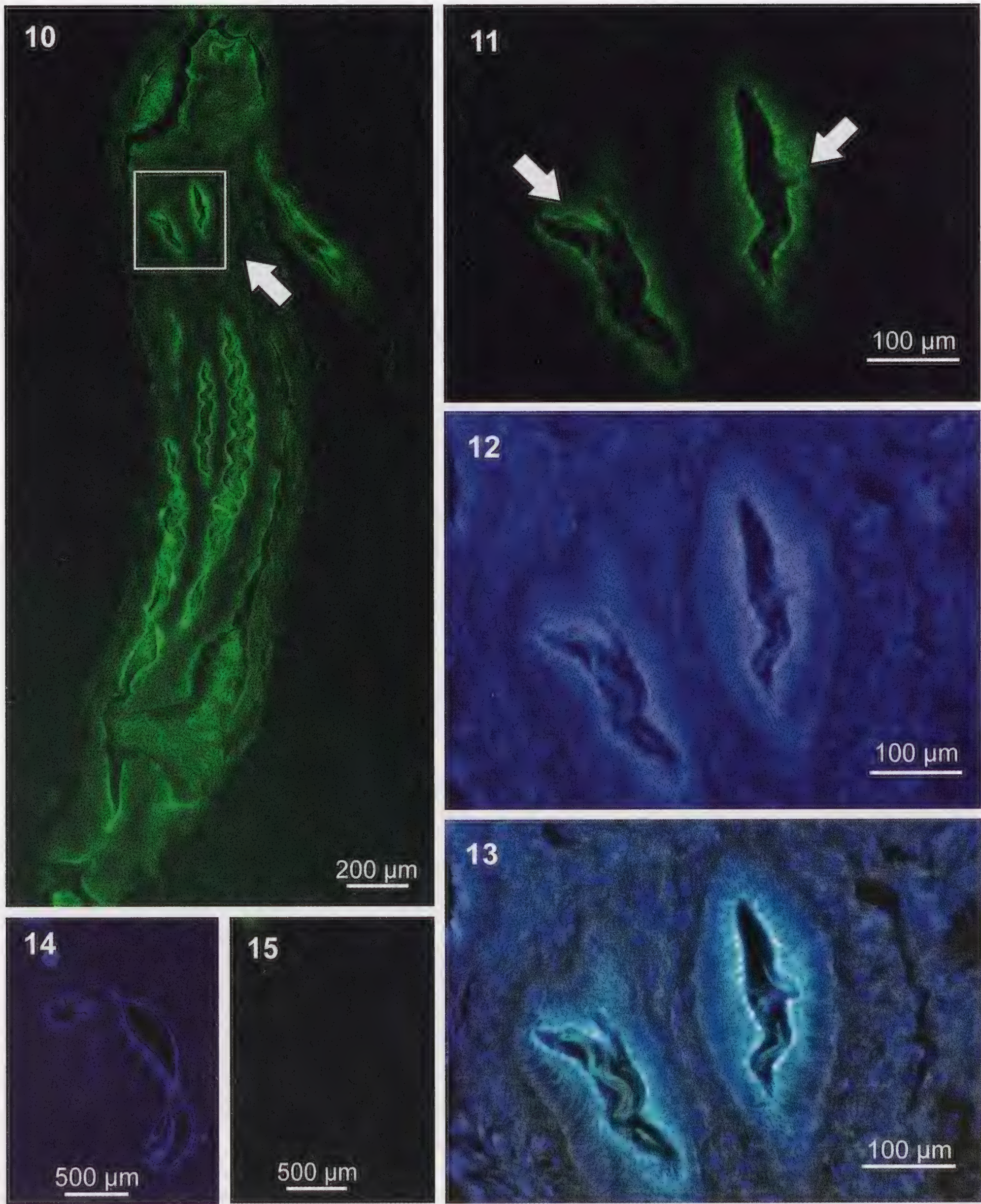
The spermatophore was obtained from two of three BrdU-treated snails that copulated for the first time and from all three snails that copulated for the second time (Table 1). One BrdU-labelled snail did not deliver a spermatophore. The percentage of labelled sperm among all sperm delivered in spermatophores of BrdU-treated snails averaged 99.3% (range: 98.3–99.9%; Table 1). The percentage of labelled sperm did not differ between snails that mated for the first time and those that mated for the second time (Mann-Whitney U-test: z = 1.70, n = 5, p = 0.21). The repeatability of the sperm counting method was 99.8% (F_{2,11} = 1502.3, p < 0.001).



FIGS. 4–9. BrdU-labelled and DAPI-stained sperm. Arrows indicate the location of one section, which is presented with higher magnification in the lower left corner of the image. FIG. 4: BrdU-labelled sperm visualized with FITC filter; FIG. 5: BrdU-labelled sperm (identical to Fig. 4) visualized with DAPI filter; FIG. 6: Unlabelled sperm visualized with DAPI filter; FIGS. 7–9: FITC and DAPI filter images are overlaid; FIG. 7: BrdU-labelled sperm; FIG. 8: Unlabelled sperm; FIG. 9: Sperm mixture (1:1) from a BrdU-labelled and an unlabelled sperm donor. Two BrdU-labelled and two unlabelled sperm heads are visible.

The total number of sperm delivered in spermatophores of BrdU-labelled individuals averaged 1.28×10^6 (range: 0.96 – 1.69×10^6 ; $n = 5$; Table 1). In unlabelled control individuals, the total number of sperm delivered aver-

aged 1.13×10^6 (range: 0.94 – 1.48×10^6 ; $n = 6$). There was no significant difference in the number of sperm delivered between labelled and unlabelled individuals ($t = 1.00$, $df = 9$, $p = 0.29$). These results indicate that both the label-



FIGS. 10–15. BrdU-labelling and DAPI-staining of spermatheca of single mated snails. FIG. 10: Cross section of spermatheca storing BrdU-labelled sperm emitting FITC fluorescence. Boxed area marked by the arrow indicates the area enlarged in FIGS. 11–13; FIG. 11: Spermathecal tubuli visualized with FITC filter. Arrows indicate BrdU-labelled sperm attached to the walls of the tubuli; FIG. 12: Spermathecal tubuli (identical to FIG. 11) visualized with DAPI filter; FIG. 13: Overlay image of FIGS. 11, 12; FIG. 14: Spermatheca storing unlabelled sperm visualized with DAPI-filter; FIG. 15: negative control: spermatheca storing unlabelled sperm visualized with FITC filter; no autofluorescence was visible.

ling method and the timing of BrdU-application are appropriate to achieve a high rate of sperm labelling without compromising sperm production (Figs. 4–6).

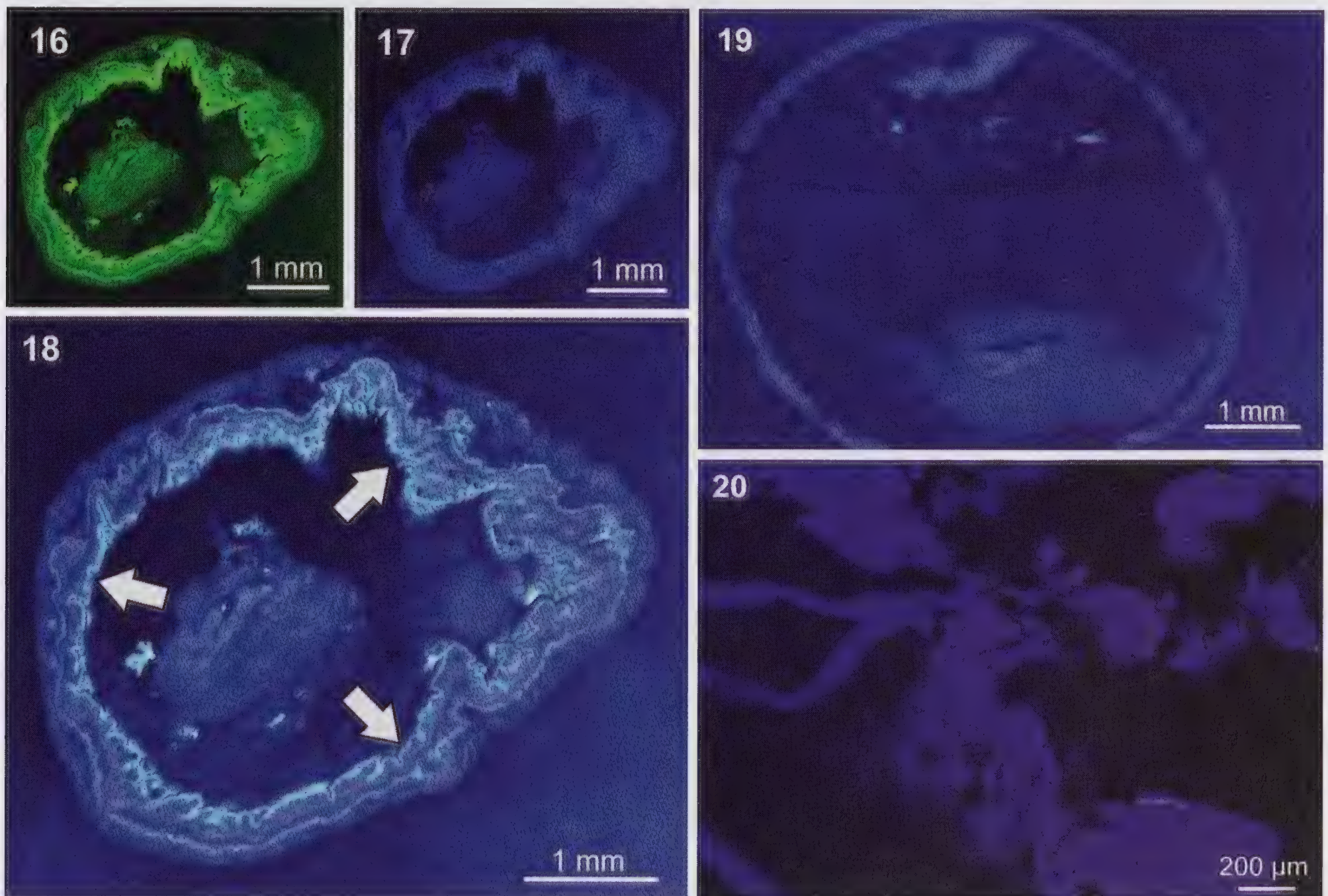
Sperm of a BrdU-labelled partner can be distinguished from sperm of an unlabelled partner (Figs. 7, 8). Overlaying BrdU/FITC- and DAPI-images allows for a count of the number of sperm heads of both types on single slides (Fig. 9). Based on a series of images the relative frequencies of sperm from either donor can be estimated (see below).

BrdU-Labelled and Unlabelled Sperm in the Female Organs of the Recipient

The spermatheca and bursa copulatrix of six unlabelled virgin snails that copulated with BrdU-treated individuals were checked for labelled sperm. All of the examined recipients stored BrdU-labelled sperm in the spermatheca

(Figs. 10–13). In control snails that mated with unlabelled individuals, sperm received could be visualized when stained with DAPI (Fig. 14), but not when stained with BrdU/FITC (Fig. 15), indicating no signs of auto-fluorescence. All snails that copulated with a BrdU-labelled partner had partly digested labelled sperm in the bursa copulatrix (Figs. 16–18). Similarly, control snails that copulated with unlabelled individuals showed partly digested unlabelled sperm in their bursa copulatrix (Figs. 19, 20). These results demonstrate that BrdU-labelled sperm entered the female reproductive organs of the recipients.

Figures 21–24 show a cross section of spermathecal tubules of a double-mated snail. The first mate was an unlabelled snail and the second one a BrdU-labelled individual. The time elapsed between the two copulations was 24 days. The overlay images (Figs. 23, 24) from BrdU/FITC immunolabelling and DAPI stain-



FIGS. 16–20. BrdU immunolabelling and DAPI-staining of bursa copulatrix. FIG. 16: Bursa copulatrix visualized with FITC filter, showing the presence of BrdU-labelled sperm; FIG. 17: Bursa copulatrix (identical to FIG. 16) visualized with DAPI filter; FIG. 18: Overlay image of FIGS. 16 & 17. Arrows indicate areas with sperm in the process of digestion; FIG. 19: Overlay image of FITC and DAPI filters of bursa copulatrix that only contains unlabelled sperm; FIG. 20: Overlay image of FITC and DAPI filters, showing enlarged section of bursa copulatrix that only contains unlabelled sperm.

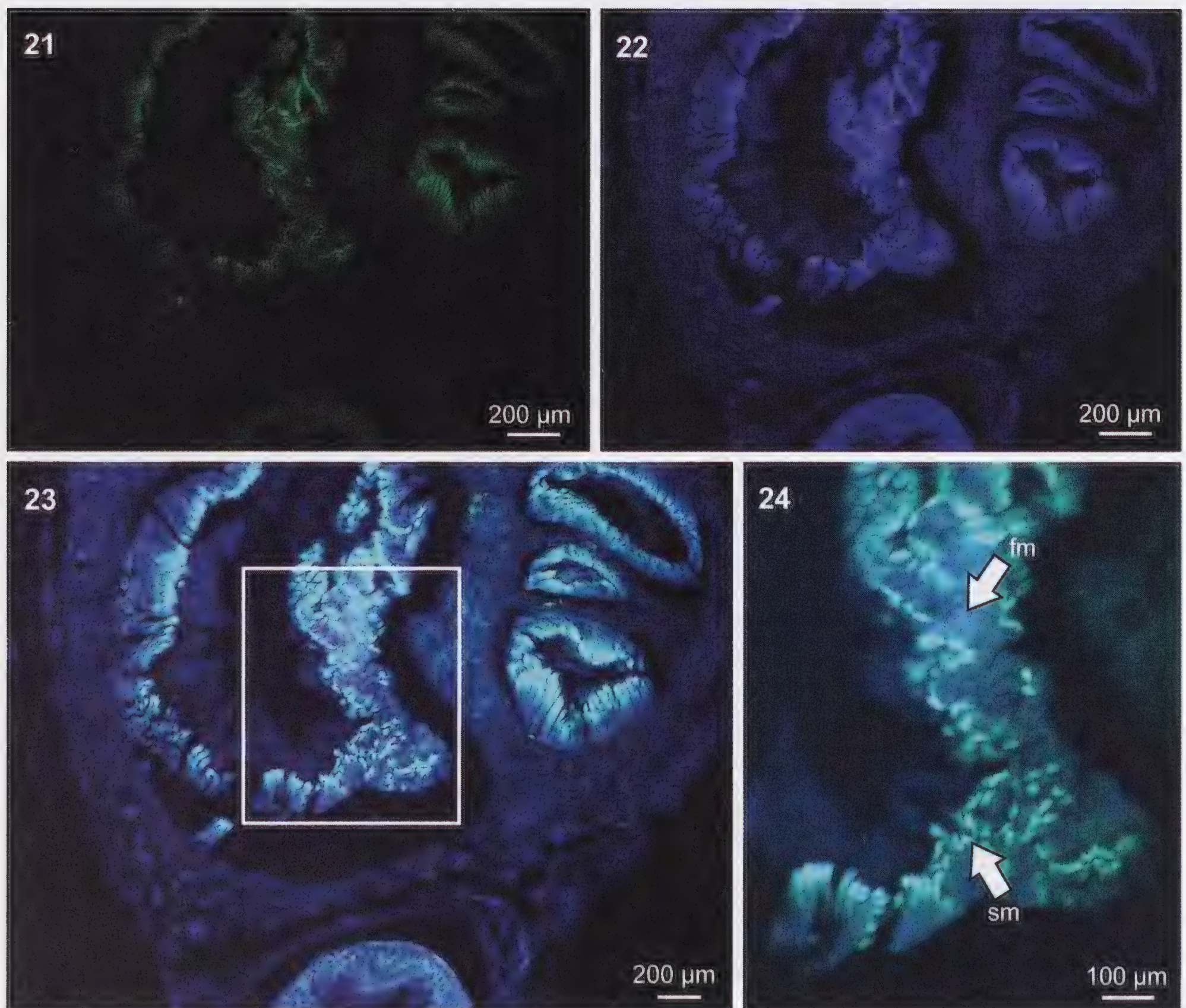
ing allow sperm from the two partners to be distinguished. Figure 23 shows a biased sperm storage. The majority of sperm stored in the main tubule were from the first mating partner. These sperm were attached to the wall of the tubule. Sperm from the second mating partner filled unoccupied parts of the spermathecal wall of the main tubule and three lateral tubules at the right upper corner of the image.

In the slice shown in Figure 23, 63% of the sperm stored were from the first mate (unlabelled sperm) and 37% from the second mate (BrdU-labelled sperm). Considering the entire spermatheca, 45% of the sperm stored were from the first mate and 55% from the second

mate (estimate based on ten slices; see Methods). The spermatheca of another double-mated snail contained 26% of the sperm from the first mate and 74% from the second mate (estimate based on ten slices).

Effect of BrdU on Snail Growth and Adult Size

Juvenile snails of the BrdU-labelled group did not differ in shell width from individuals of the control group at the beginning of the experiment (mean $\pm$ SE: 12.0 $\pm$ 0.2 mm vs. 12.1 $\pm$ 0.3 mm; $t = 0.32$, $df = 33$, $p = 0.75$). BrdU-injection seems to retard shell growth. In the first 42 days of the experiment (after three



FIGS. 21–24. BrdU-immunolabelled and DAPI-stained spermatheca of a double-mated individual. FIG. 21: Spermatheca showing multiple tubuli visualized with FITC filter; FIG. 22: Spermathecal tubuli (identical to FIG. 21) visualized with DAPI filter; FIG. 23: Overlay image of FIGS. 21 & 22. Boxed area indicates magnification shown in FIG. 24; FIG. 24: Section of main tubule showing BrdU-labelled and unlabelled sperm from two different donors. Abbreviations: *fm* first mate (with unlabelled sperm), *sm* second mate (with BrdU-labelled sperm).

injections), labelled snails had a reduced shell growth rate compared to control snails (0.073 ± 0.006 mmday⁻¹ vs. 0.098 ± 0.006 mmday⁻¹; $t = 2.90$, $df = 33$, $p = 0.0067$). The difference in growth rate became smaller but remained significant when a period of 134 days (after eight injections) was considered (0.028 ± 0.002 mmday⁻¹ vs. 0.034 ± 0.002 mmday⁻¹; $t = 2.34$, $df = 33$, $p = 0.0257$). However, long-term application of BrdU did not affect adult shell size. Juvenile snails randomly assigned either to the BrdU-treated group or the control group did not differ in adult size attained during the experiment (labelled vs. unlabelled: 17.1 ± 0.4 mm vs. 17.5 ± 0.3 mm; $t = 0.79$, $df = 13$, $p = 0.44$). In each group, one snail died during the experiment, suggesting that BrdU-injection did not increase mortality in *A. arbustorum* (survival of labelled vs. unlabelled: 94.4% vs. 94.1%; $X^2 = 0.002$, $df = 1$, $p = 0.96$).

Five BrdU-treated and nine control snails reached sexual maturity within 100 days. These snails were used in mating trials. Three of the five BrdU-treated snails mated (60.0%) as did six of the nine control snails (66.7%), indicating that there is no difference in mating propensity between the two groups of snails ($X^2 = 0.062$, $df = 1$, $p = 0.80$).

DISCUSSION

We presented a sperm-labelling technique that allows, in combination with a traditional staining method, the tracking of sperm from two donors (one with labelled sperm, the other with unlabelled sperm) in the reproductive organs of double-mated recipients in hermaphroditic gastropods. A series of tests demonstrate a high efficiency of the sperm-labelling procedure and show that labelled sperm can be visualized both in the sperm storage and sperm digesting organs of the recipient with a high reliability. This immunocytochemical approach opens new ways to investigate mechanisms and processes of postcopulatory sexual selection, including sperm transfer and both selective sperm storage and sperm utilization.

In the past, studies dealing with sperm distribution in the reproductive organs of the recipient utilising traditional histological staining methods could not distinguish between sperm from different mates. Moreover, sperm of radiolabelled males could not be precisely localized in the female reproductive tract (Bishop & Sommerfeldt, 1996). Most recently, insertions of fluorescence

genes were used to distinguish sperm of different males in the female tract in *D. melanogaster* (by Manier et al., 2010). However, such transgenic transformations require a detailed knowledge of the species genome for a successful application, which is so far available only for a few species. There is evidence that the degree of genetic heterozygosity of an individual influences its mating activity and reproductive success in *A. arbustorum* (by Minorette et al., 2011). If postcopulatory female choice (sperm storage or digestion) is based on the genetic heterozygosity or complementarity of mates, then competitive PCR is of limited use. In contrast, the improved immunostaining technique presented here may allow unbiased detection of distinctively labelled sperm in the female tract in a variety of gastropod species. Combined with traditional sperm staining methods, the immunostaining technique allows to distinguish between sperm from two males. It could even be further developed by including a second sperm labelling substance (Miller et al., 1991; Schärer et al., 2007). Furthermore, it could be adjusted to other invertebrate species.

In studies of postcopulatory selection, the application of BrdU should be well timed because the synthetic analogue is only incorporated into newly synthesized nucleic acids. To achieve a high rate of sperm labelling success a repeated or continuous application during growth and developmental of testes or ovotestis of the individuals as well as during sperm production is required. In a series of tests we could demonstrate a high rate of BrdU-labelling in sperm of *A. arbustorum*.

Long-term application of BrdU increases the risk of cell changes which may lead to cell death (Caldwell et al., 2005). Application of BrdU may also affect growth and reproduction. We found that repeated BrdU-injections retarded the shell growth of juvenile *A. arbustorum*, but did not influence the adult shell size attained and the number of sperm delivered in spermatophores. Furthermore, we did not record increased mortality or a decreased mating frequency in BrdU-treated individuals compared with control snails. Moreover, the distribution of BrdU-labelled sperm found in the sperm storage organ of the recipients did not differ from that of unlabelled sperm stained with traditional histological methods reported in previous studies (Baminger & Haase, 1999; Bojat et al., 2001b). This indicates that BrdU-labelling has some effects on the physiology of snails, but does not influence the male reproductive behaviour and sperm number in *A. arbustorum*. Thus, BrdU-

labelling may be a useful tool to study the fate of sperm in the female reproductive organs of the recipient. Combined with DAPI staining, immunostaining of BrdU-labelled sperm allows the investigation of sperm distribution in double-mated snails, i.e. BrdU-labelled vs. unlabelled sperm in the recipient.

We also presented a method to estimate the relative amount of sperm stored from each of the two donors in the spermatheca of the recipient. This estimate allows a comparison between the proportion of sperm stored and the percent of paternity (P_2 -value) in previously produced egg batches. Furthermore, this method allows estimates of the percentage of sperm stored in the main tubule and in each lateral tubule and thus to study the mechanism of sperm storage in double-mated snails.

We also showed that the sperm-labelling technique could be used to investigate the mechanisms of sperm digestion which might allow to follow digested particles originating from the labelled sperm in the recipient's tissue (e.g., to examine whether nucleic acids of digested sperm and spermatophores are incorporated in newly produced eggs). However, it should be kept in mind that the technique presented is limited to a snapshot in time, because of the tissue fixation to achieve fluorescence detection of labelled sperm. Consequently, dynamic processes in the sperm storage organ cannot be followed with this labelling technique.

In summary, the use of BrdU for sperm labelling has many applications in *A. arbustorum* and it could be adjusted to a variety of other gastropod and invertebrate species. With this technique, the fate of sperm can be followed in the female reproductive tract after copulation. In double-mated individuals, the sperm-labelling technique can give insight into mechanisms of postcopulatory sexual selection (Beese et al., 2006b). This technique may improve our understanding of the evolution of male and female morphology and physiology and of postcopulatory selection in general in hermaphroditic and gonochoristic species.

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